AD_____

GRANT NO: DAMD17-94-J-4311

TITLE: Microenvironment of Breast Tissue: Lithocholic Acid and Other Intestinal Steroids

PRINCIPAL INVESTIGATOR(S): Norman B. Javitt, M.D., Ph.D.

CONTRACTING ORGANIZATION: New York University Medical Center
New York, New York 10016

REPORT DATE: September 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1995	Annual 1 Sep 94 - 31 Aug 95

4. TITLE AND SUBTITLE
Microenvironment of Breast Tissue: Lithocholic Acid and Other Intestinal Steroids

5. FUNDING NUMBERS
DAMD17-94-J-4311

6. AUTHOR(S)
Norman B. Javitt, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
New York University Medical Center
New York, New York 10016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Methods are in development for the analysis of bile acids in breast cyst fluid. For detection of bile acids at the femtomole level as a fluorescent ester linked through the 3-hydroxyl group common to most naturally-occurring bile acid, pyrene-1-carbonyl azide is under evaluation. Using known bile acid standards the fluorescent derivatives have been prepared under mild conditons and separated by reverse phase HPLC. However, decomposition of the azide and the instability of the isocyanate formed during the reaction gives a number of interfering fluorescent peaks. Fractionation of breast cyst fluid using a reverse phase C-18 cartridge provides an aliquot containing bile acids. However, unidentified fluorescent peaks also occur which may represent either artifacts or unidentified naturally-occurring compounds in breast cyst fluid. Resolution of the remaining analytical problems will permit analysis of 99 samples of breast fluid that have been obtained thus far.

14. SUBJECT TERMS	15. NUMBER OF PAGES
microenvironment of breast tissue, breast cyst fluid, lithocholic acid, intestinal steroids Breast Cancer	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to ***stay within the lines*** to meet ***optical scanning requirements***.

Block 1. Agency Use Only *(Leave blank)*.

Block 2. Report Date. Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

Block 3. Type of Report and Dates Covered. State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

Block 4. Title and Subtitle. A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

Block 5. Funding Numbers. To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C	- Contract	**PR**	- Project
G	- Grant	**TA**	- Task
PE	- Program Element	**WU**	- Work Unit Accession No.

Block 6. Author(s). Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

Block 7. Performing Organization Name(s) and Address(es). Self-explanatory.

Block 8. Performing Organization Report Number. Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es). Self-explanatory.

Block 10. Sponsoring/Monitoring Agency Report Number. *(If known)*

Block 11. Supplementary Notes. Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

Block 12a. Distribution/Availability Statement. Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

DOD	- See DoDD 5230.24, "Distribution Statements on Technical Documents."
DOE	- See authorities.
NASA	- See Handbook NHB 2200.2.
NTIS	- Leave blank.

Block 12b. Distribution Code.

DOD	- Leave blank.
DOE	- Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.
NASA	- Leave blank.
NTIS	- Leave blank.

Block 13. Abstract. Include a brief *(Maximum 200 words)* factual summary of the most significant information contained in the report.

Block 14. Subject Terms. Keywords or phrases identifying major subjects in the report.

Block 15. Number of Pages. Enter the total number of pages.

Block 16. Price Code. Enter appropriate price code *(NTIS only)*.

Blocks 17. - 19. Security Classifications. Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

Block 20. Limitation of Abstract. This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

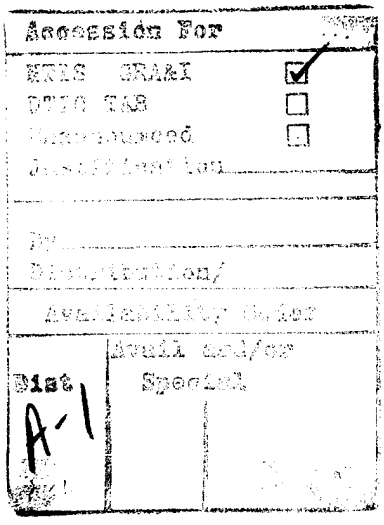
____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

__X__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



Laura B. Lavett 9/14/95
PI - Signature Date

TABLE OF CONTENTS

5. INTRODUCTION

Previous studies established the presence of high concentrations of bile acids in breast cyst fluid. The procedures that were used, particularly gas-liquid chromatography, required the preparation of volatile derivatives and by necessity, therefore, the use of chemical procedures that preclude the possibility of identifying the actual chemical state of the bile acid in the breast cyst fluid. Thus, it is not known if the lithocholic acid found in many samples of breast cyst fluid is actually present as either the taurine or glycine conjugate and also if it is esterified at the 3 position with glucuronic acid or as a sulfate. Thus, lithocholic acid could be present in breast cyst fluid in as many as 9 different chemical species (lithocholic acid, the 3-glucuronide or 3-ester sulfate, the glycine and taurine conjugates, either free or esterified with glucuronic acid or sulfate). Similarly other bile acids that are present could also have a minimum of 9 different species if one neglects the possibility that chenodeoxycholic acid may be esterified at either the 3 or 7 position.

To begin to tackle this analytical problem it was proposed that newer methods that would permit identification of the actual species that exists in breast cyst fluid be developed. This determination is important because it is known that each of the species of bile acids can have different biologic effects **[1]** and therefore a closer insight on the relationship to breast cancer can be obtained by identifying the actual species of bile acid present.

6. BODY: EXPERIMENTAL METHODS AND RESULTS

As indicated in the grant application our approach is to develop fluorescent derivatives of the bile acids that can be analyzed by HPLC.

REAGENT SELECTION: After review of the literature **[2,3]** we chose to evaluate pyrene-1-carbonyl azide (PCA) **[4]**. This reagent was chosen because:

a. the pyrene structure is likely to give the highest sensitivity for detection of the derivatives.

b. the formation of an adduct at the 3-hydroxy position common to all bile acids would detect all (1) unconjugated bile acids, (2) glycine-conjugated bile acids, and (3) taurine-conjugated bile acids. Bile acids esterified at the 3-position, such as glucuronides or sulfates, would not be detected. However, mild enzymatic treatment of the sample with β-glucuronidase and/or a sulfatase would selectively yield a free 3-hydroxyl group for formation of the PCA derivative. Thus virtually all the known species of bile acids that might be expected in breast cyst fluid could be detected.

PREPARATION OF THE REAGENT: Appendix page 1 indicates the method of synthesis of PCA from the commercially available acid. The

pyrene azide, kept in the refrigerator at -20°C underwent significant decomposition within a month.

PREPARATION OF DERIVATIVES: Appendix pages 2 and 3 indicate the reactions that occur in the formation of the PCA-3-ester derivative of sterols or bile acids. Reflux of PCA in benzene quickly converts the azide to the isocyanate. The isocyanate reacts with primary or secondary hydroxyl groups to form the pyrene ester. Although hydroxyl groups on the 7 and 12 position of the bile acid ring could theoretically form an ester, they are sterically much more hindered than at the 3 position.

ANALYSIS OF PYRENE ESTERS OF BILE ACIDS: Appendix pages 4, 5, and 6 indicate the yields of free and conjugated bile acids. It was found necessary to methylate the carboxyl group to obtain good reaction conditions. Also the presence of dichloromethane was found to greatly enhance ester formation. With 10-fold excess of PCA the reaction was virtually complete.

PREPARATION OF BREAST CYST FLUID FOR DERIVATIZATION: Appendix page 7 outlines the scheme for isolating the bile acids free of interfering substances by using a C-18 reverse phase column.

ANALYSIS OF BREAST CYST FLUID ENRICHED WITH BILE ACID STANDARDS: Appendix page 8 indicates the recovery of bile acids added to an aliquot of breast cyst fluid. Although recovery was satisfactory, the method of sample preparation gave too many peaks that overlap with the bile acid peaks.

The causes for these peaks are currently under investigation. In part they appear to be derived from (1) decomposition products of the PCA reagent, (2) impurities present in the C-18 packing, and (3) unknown naturally occurring compounds present in breast cyst fluid.

7. CONCLUSIONS

Although pyrene-1-carbonyl azide fulfills the criterion for sensitivity and provides a technique for forming a derivative via the free 3-hydroxyl group common to most of the naturally occurring bile acids, its use has a number of limitations. The reagent itself appears to be unstable, requiring frequent synthesis. The isocyanate is more unstable, needs to be freshly prepared, and yields a number of fluorescent decomposition products. For reasons that are not entirely clear esterification of the carboxyl group appears to be necessary for PCA-adduct formation to occur.

Other analytical problems relate to the isolation of the bile acid fraction from breast fluid. The use of a C-18 reverse-phase column has been a major advance in bile acid analysis. It is widely used to isolate bile acids from serum, urine, and feces. The presence, therefore, of so many unexplained peaks after derivative formation is surprising.

We are currently evaluating other fluorescent reagents and other methods of sample preparation.

We currently have 99 samples of breast cyst fluid, lyophilized and stored at -80°C awaiting bile acid analysis.

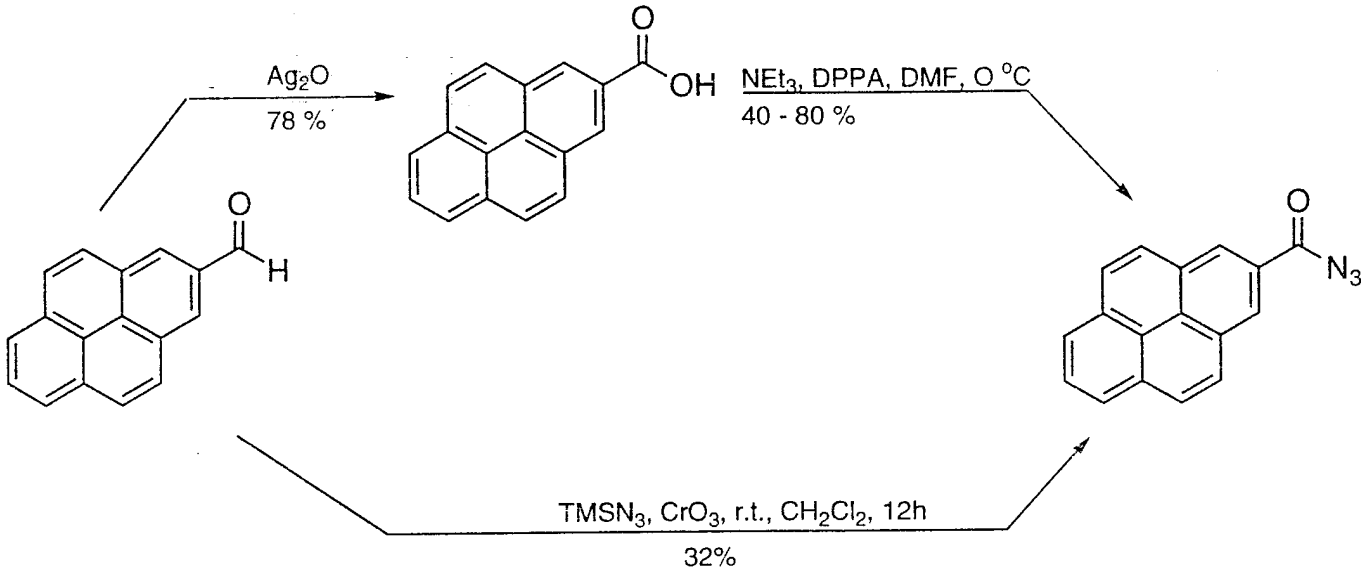
8. REFERENCES

1. Baker, P. R., Wilton, J. C., Jones, C. E., Stenzel, D. J., Watson, N., and Smith, G.J. Bile acids influence the growth, oestrogen receptor and oestrogen-regulated proteins of MCF-7 human breast cancer cells. Br. J. Cancer 65: 566-572, 1992

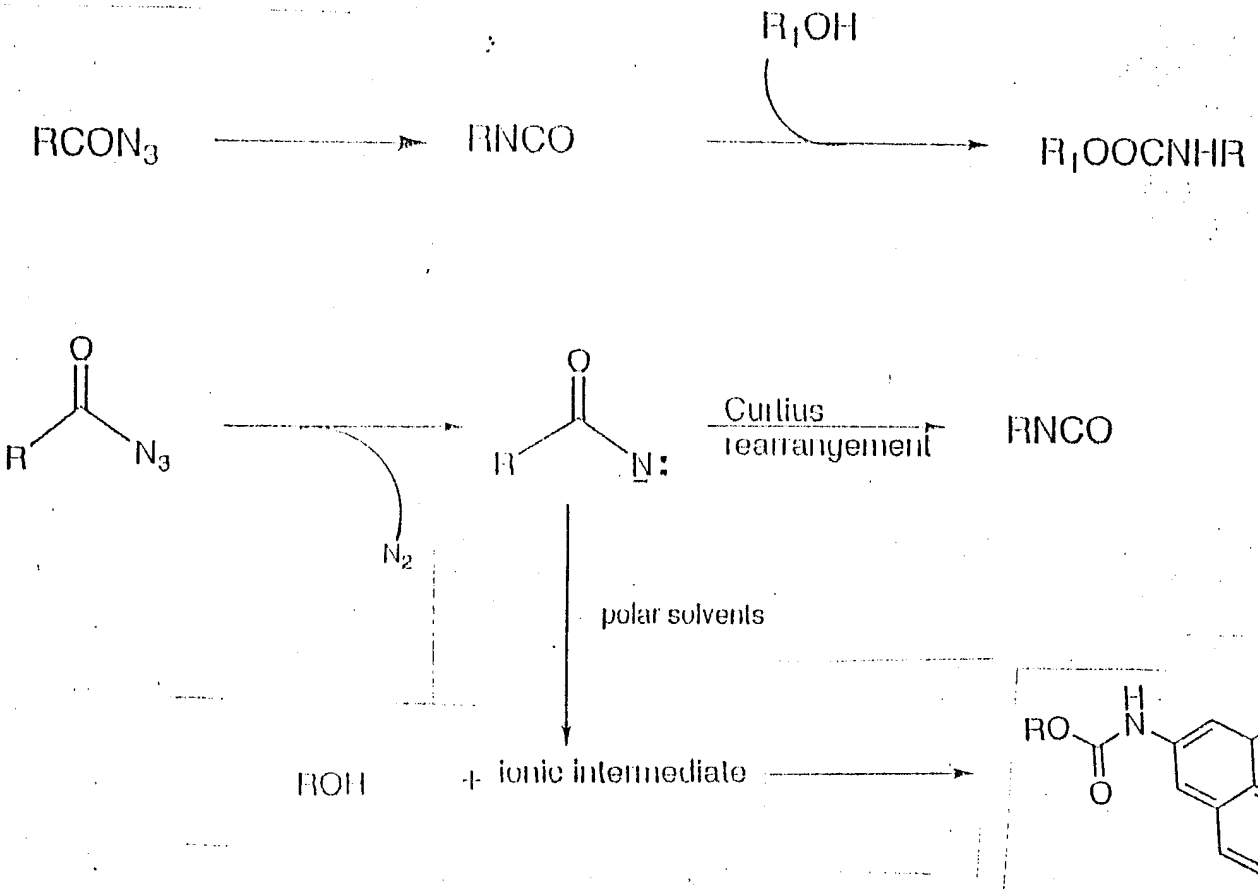
2. Sjovall, J., and Setchell, K. D. R. Techniques for extraction and group separation of bile acids. In The Bile Acids, Chemistry, Physiology, and Metabolism, Vol. 4, K. D. R. Setchell, D. Kritchevsky and P. P. Nair, Eds., Plenum Press, New York, 1988.

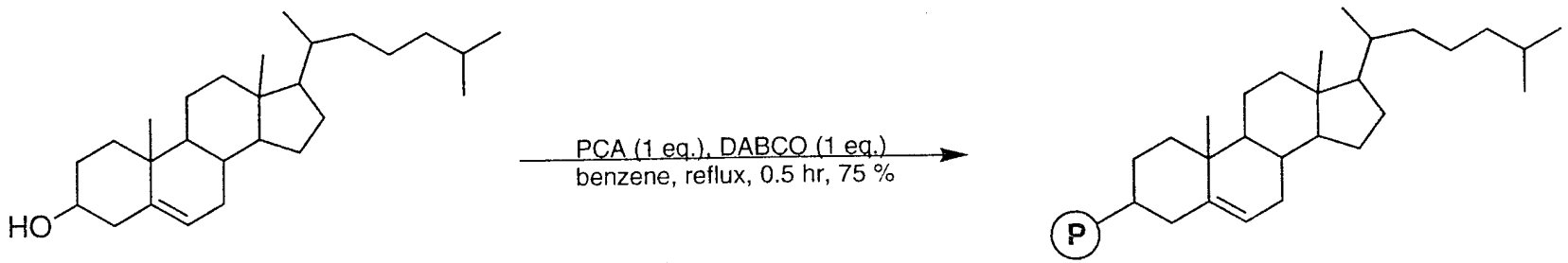
3. Nambara, T., and Goto, J. High Performance Liquid Chromatography. In The Bile Acids, Chemistry, Physiology, and Metabolism, Vol 4., K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, Eds., Plenum Press, New York, 1988

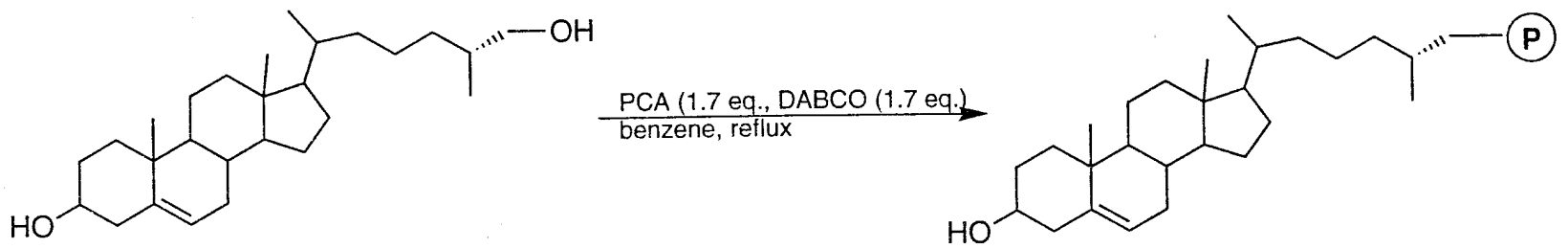
4. Fujino, H., Takeda, M. and Shujiro, G. Synthesis and reactivity of Pyrene-1-carbonyl azide as a fluorescent derivatization reagent for alcohols. Yakugaku Zasshi 110:457-461, 1990

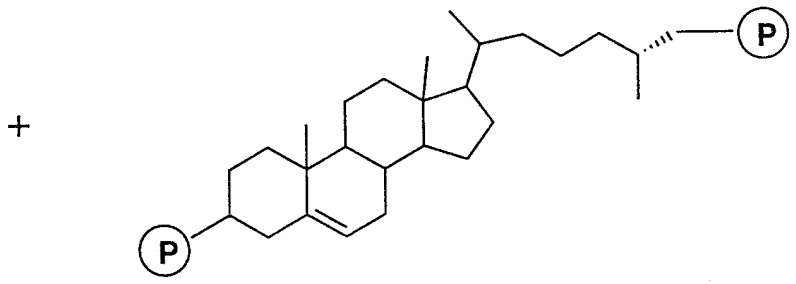


DPPA = $(PhO)_2P(O)N_3$

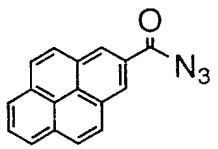

R1OH
RCON3
RNCO
R1OOCNHR
O
R
N3
N2
O
R
N:
Curtius
rearrangement
RNCO
polar solvents
ROH
+ ionic intermediate
RO
H
N
O


HO
PCA (1 eq.), DABCO (1 eq.)
benzene, reflux, 0.5 hr, 75 %
P

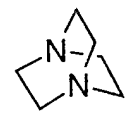

OH
HO
PCA (1.7 eq., DABCO (1.7 eq.)
benzene, reflux
P
HO


+
P

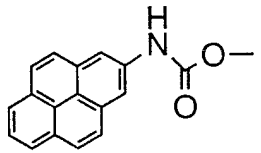
PCA =

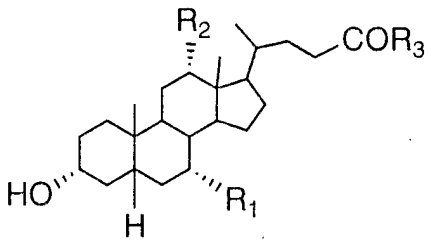

O
N3

DABCO =

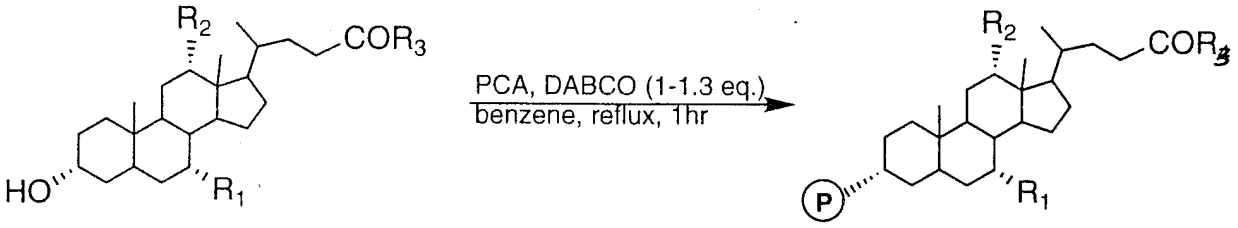

N
N

P =

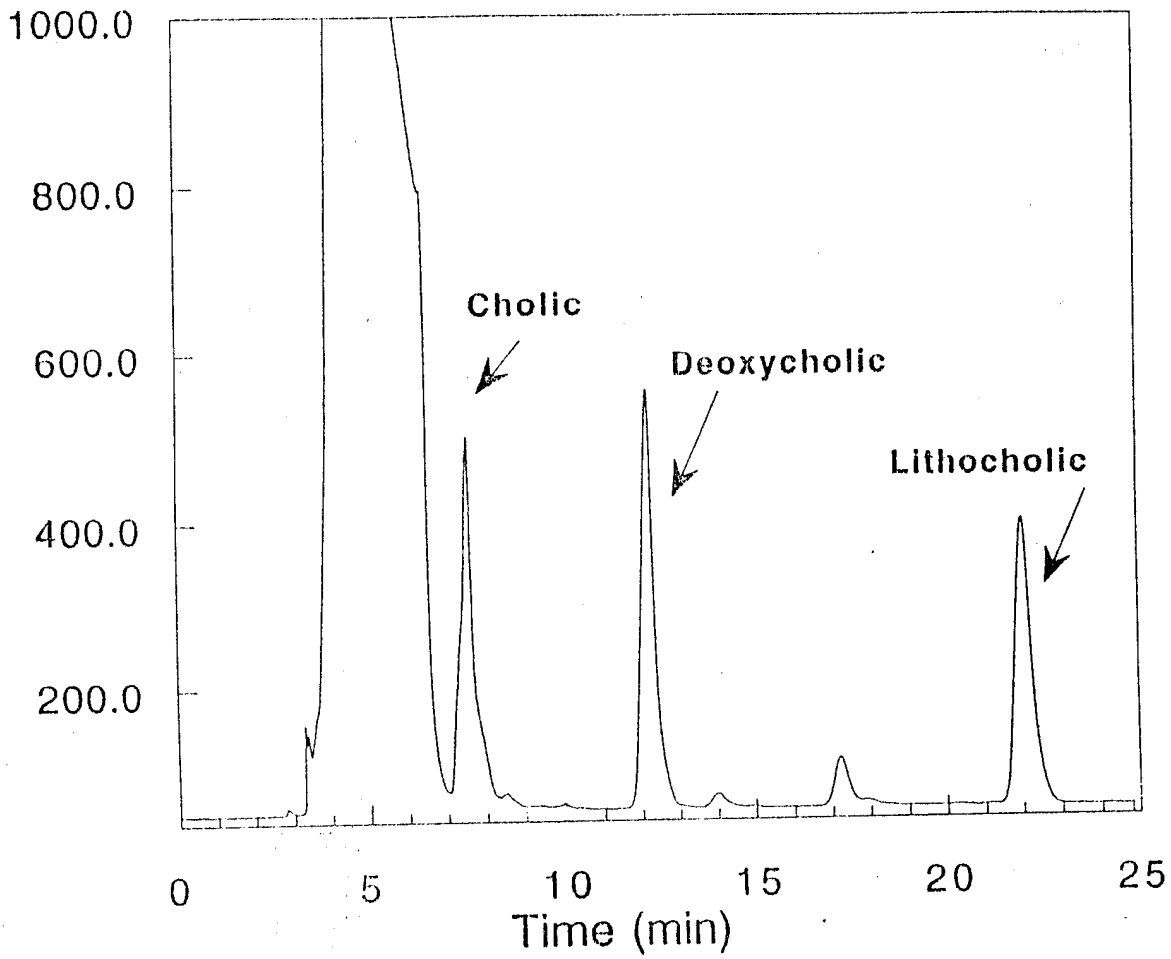

H
N
O
O



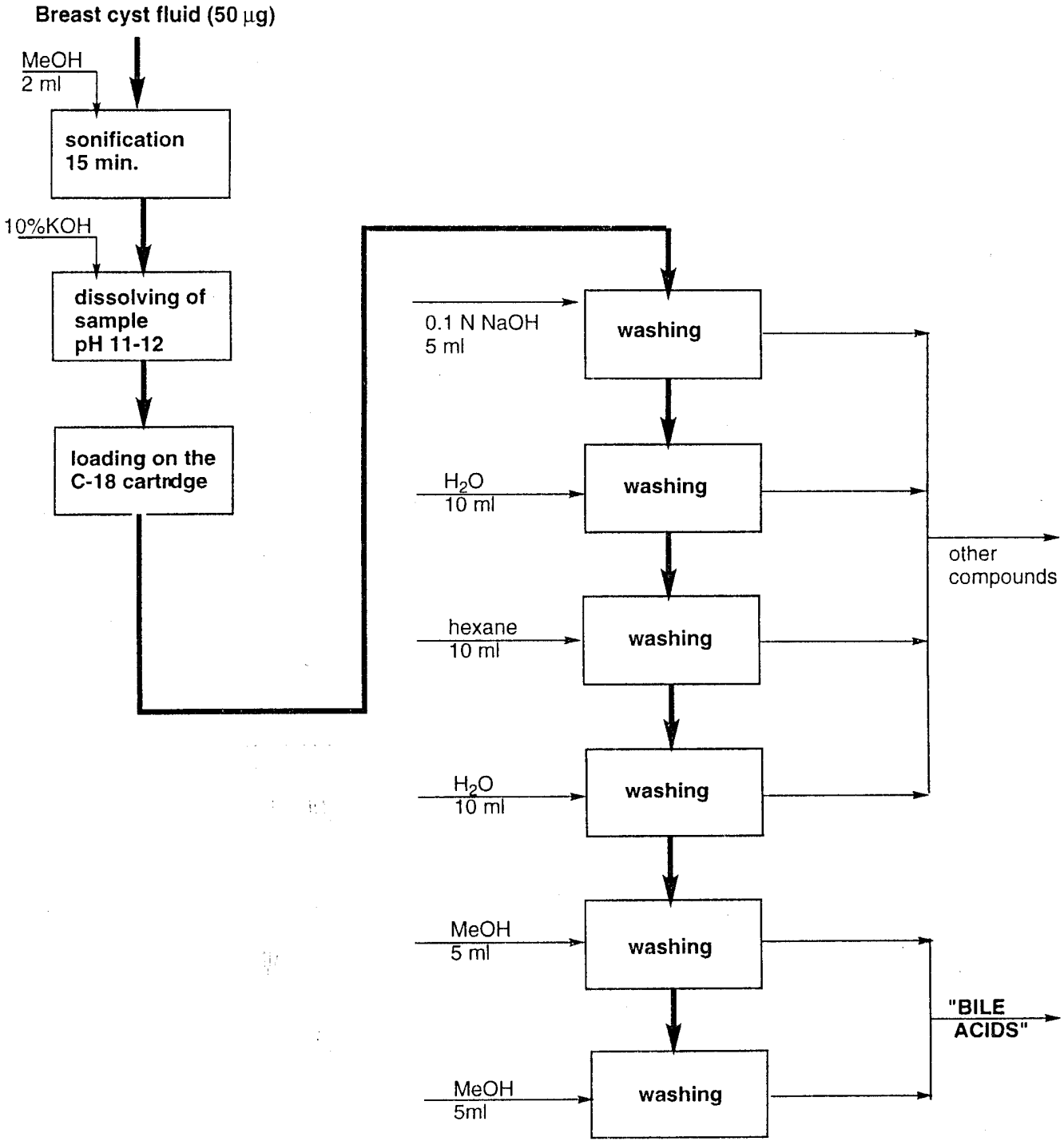
R_1	R_2	R_3	name of acid
OH	OH	OH	cholic
H	OH	OH	deoxycholic
OH	H	OH	chenodeoxycholic
H	H	OH	lithocholic
OH	OH	$NHCH_2CH_2COOH$	glycocholic
OH	OH	$NHCH_2CH_2SO_3H$	taurocholic



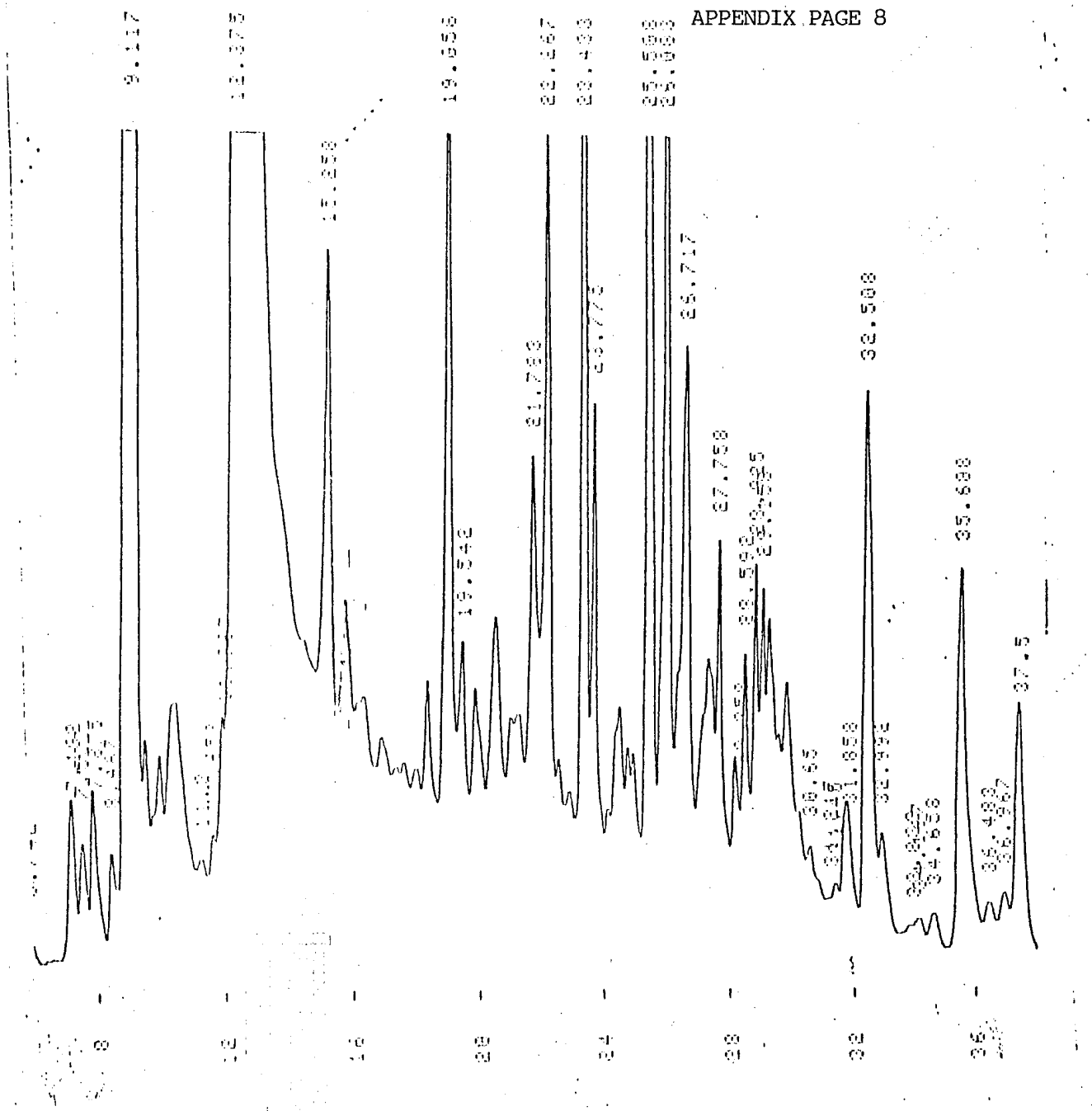
Entry	R_1	R_2	R_3	Yield of derivative
1	H	H	COOMe	22
2	H	OH	COOMe	81
3	OH	H	COOMe	not determined
4	OH	OH	COOMe	19
5	H	H	$NHCH_2COOMe$	
6	H	OH	$NHCH_2COOMe$	
7	OH	H	$NHCH_2COOMe$	42
8	OH	OH	$NHCH_2COOMe$	44
9	OH	OH	$NHCH_2CH_2SO_3Me$	not determined



Conditions: 2 nmol (2×10^{-9} mol) of each methyl ester of bile acid, PCA 100 nmol (1×10^{-7} mol) and DABCO 120 nmol (1.2×10^{-7} mol) in 200 µl of CH_2Cl_2 were heated in 60 °C until complete evaporation of solvent. Then 100 µl of CH_2Cl_2 was added and 5 µl of aliquot were injected.



Preparation breast cyst fluid sample for derivatization



RECOVERY OF BILE ACID STANDARDS ADDED TO BREAST CYST FLUID
HPLC OF PCA DERIVATIVES AFTER ELUTION FROM C-18 COLUMN